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Supplemental Information

Outer Membrane Vesicles of a Human Commensal Mediate Immune Regulation and Disease Protection

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SUPPLEMENTAL INVENTORY

Figure S1, related to Figure 1. Wild-type *B. fragilis* and *B. fragilis*ΔPSA deletion mutant produce similar amounts of OMVs and have similar proteomic profiles.

Figure S2, related to Figure 2. PSA-containing OMVs given orally to animals suppress pro-inflammatory cytokines and induce anti-inflammatory cytokines in colon tissue during TNBS colitis.

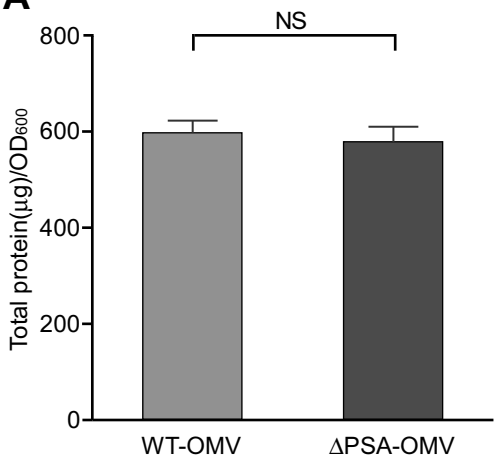
Figure S3, related to Figure 3. OMVs are internalized and localized in the cytoplasm of DCs, and up-regulate co-stimulatory molecules; neutralization of IL-10R signaling partially abrogates PSA activity *in vitro*.

Figure S4, related to Figure 4. TLR2-/- DCs internalize OMVs, and purified PSA elicits IL-10 production from T cells in a TLR2-dependent manner, while PSA-containing OMVs do not induce IL-10 directly from T cells.

Figure S5, related to Figure 5. Gadd45α-/- DCs internalize OMVs, and Gadd45α is required in BMDCs to mediate PSA activity during protection from intestinal inflammation.

SUPPLEMENTAL DATA

A

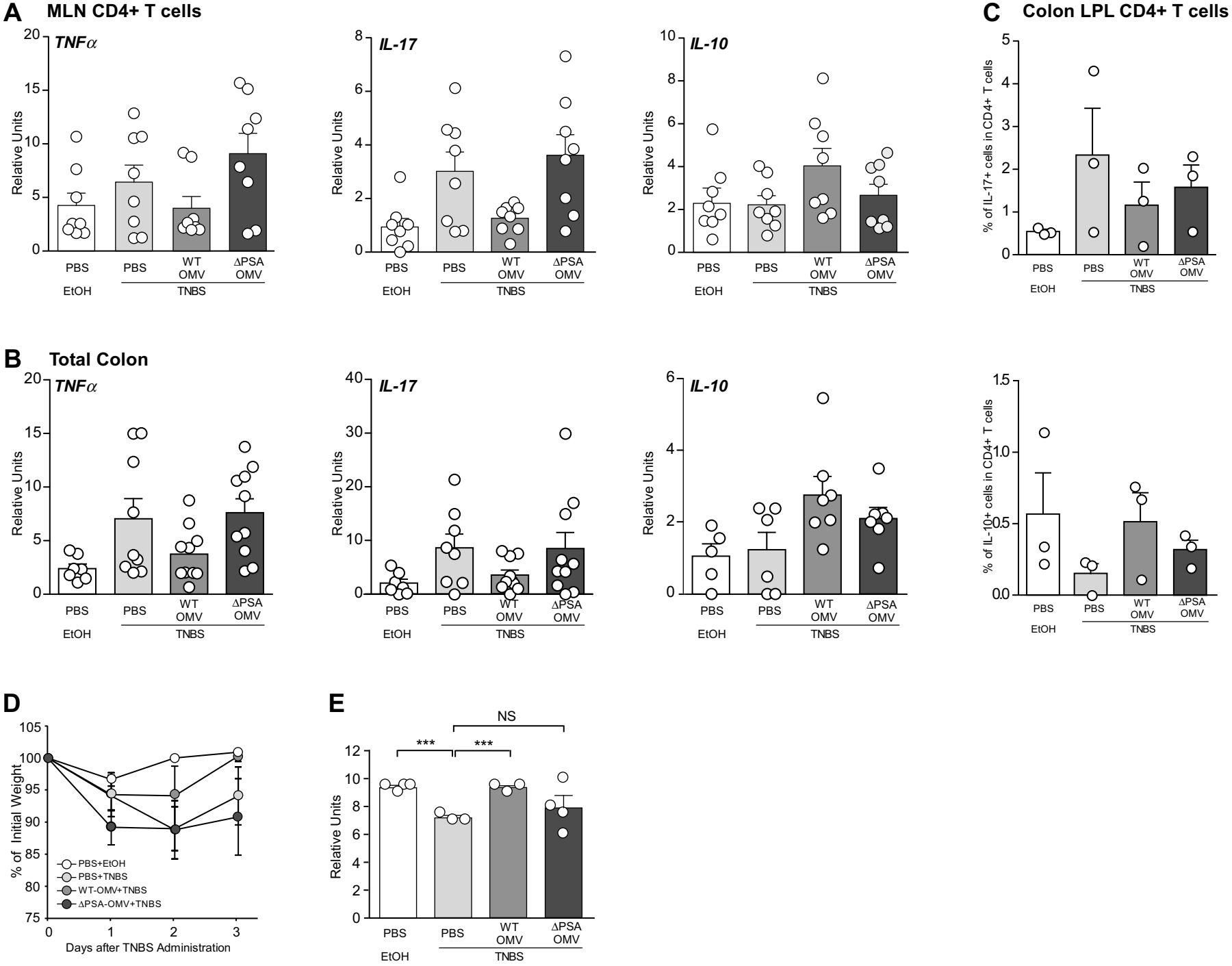


B

No.	Accession No.	Protein Name	WT-OMV	ΔPSA-OMV
BF3567	gi|60683022	hypothetical protein	864±58	452±65
BF2157	gi|60681636	putative lipoprotein	647±45	630±37
BF0595	gi|60680161	hypothetical protein	341±25	296±18
BF2161	gi|60681640	hypothetical protein	213±24	85±5
BF2706	gi|60682179	putative lipoprotein	178±18	128±12
BF1956	gi|60681445	putative outer membrane protein	161±41	129±9
BF0594	gi|60680160	hypothetical protein	142±12	202±8
BF1957	gi|60681446	hypothetical protein	134±10	157±17
BF3067	gi|60682536	putative lipoprotein	124±31	62±6
BF0589	gi|60680155	hypothetical protein	124±17	119±12
BF3432	gi|60682894	hypothetical protein	117±9	96±11
BF2023	gi|60681124	putative ATP/GTP-binding protein	117±13	74±1
BF1619	gi|60681115	hypothetical protein	117±9	147±14
BF3144	gi|60682613	putative lipoprotein	107±22	110±14

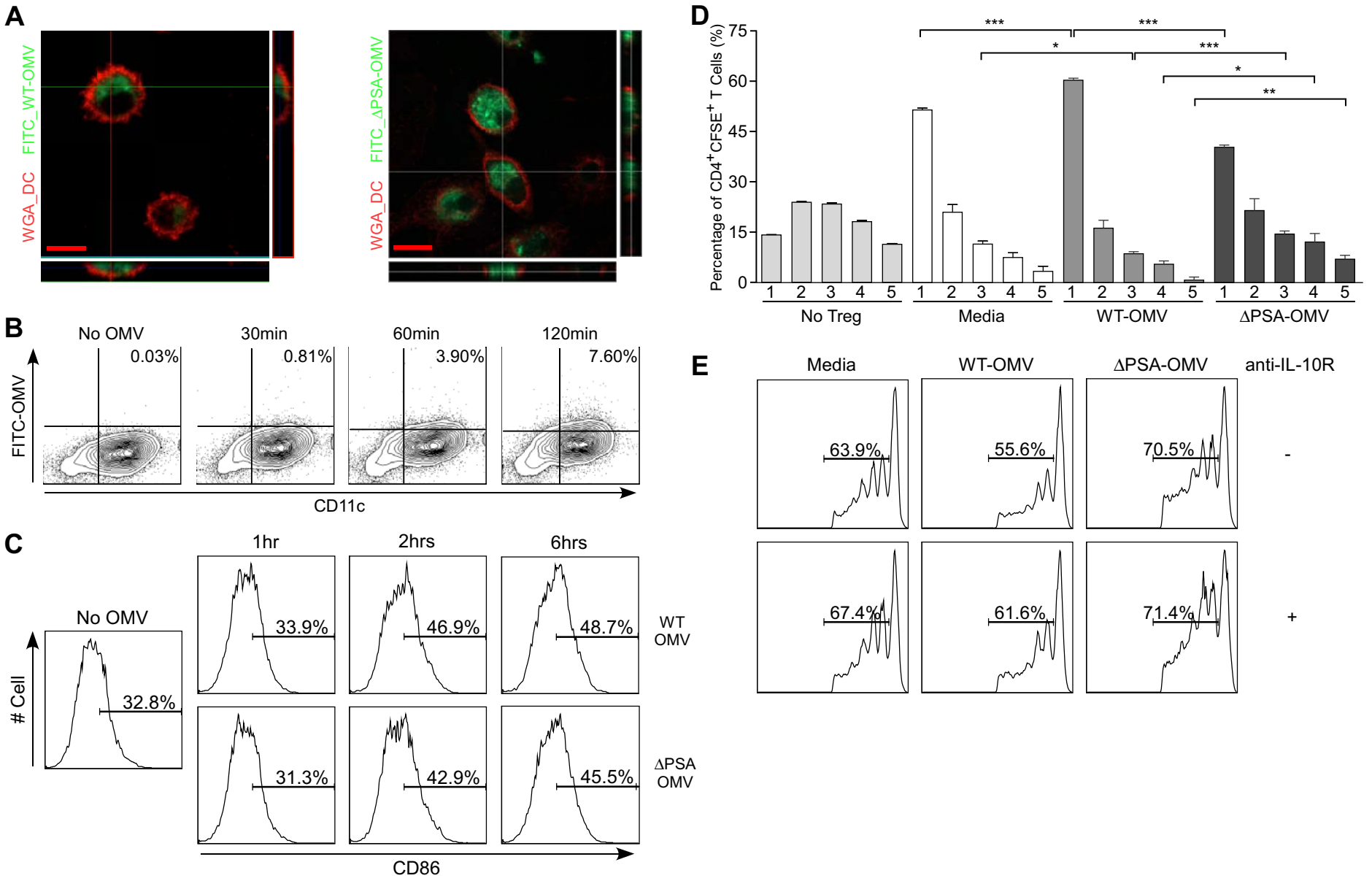
Shen *et al.*, Supplemental Figure 1

Figure S1, related to Figure 1. Wild-type *B. fragilis* and *B. fragilis*ΔPSA deletion mutant produce similar amounts of OMVs and have similar proteomic profiles. (**A**)Wild-type *B. fragilis* and the PSA deletion mutant (*B. fragilis*ΔPSA) produce similar amounts of OMVs during *in vitro* culture. Total protein recovered from each OMV preparation was normalized by OD_{600} of the culture at the time of harvest. Error bars indicate SEM. Result is shown from >10 combined experiments performed independently. *p* value determined by Student's t-test. NS: not significant. (**B**) OMVs from wild-type or PSA deletion mutant *B. fragilis* show no significant difference in protein composition. Proteome mass spectrometry shows 100% overlap of the identified proteins (>1 unique peptide identified for each protein) between WT-OMVs and ΔPSA-OMVs. Among all of the identified proteins, we semi-quantitatively compared the amount of those relatively abundant proteins according to the number of unique peptides identified. The majority of them show no difference between WT-OMV and ΔPSA-OMV. Last two columns represent number of unique peptides ±SEM. Results are shown from 3 combined experiments performed independently.



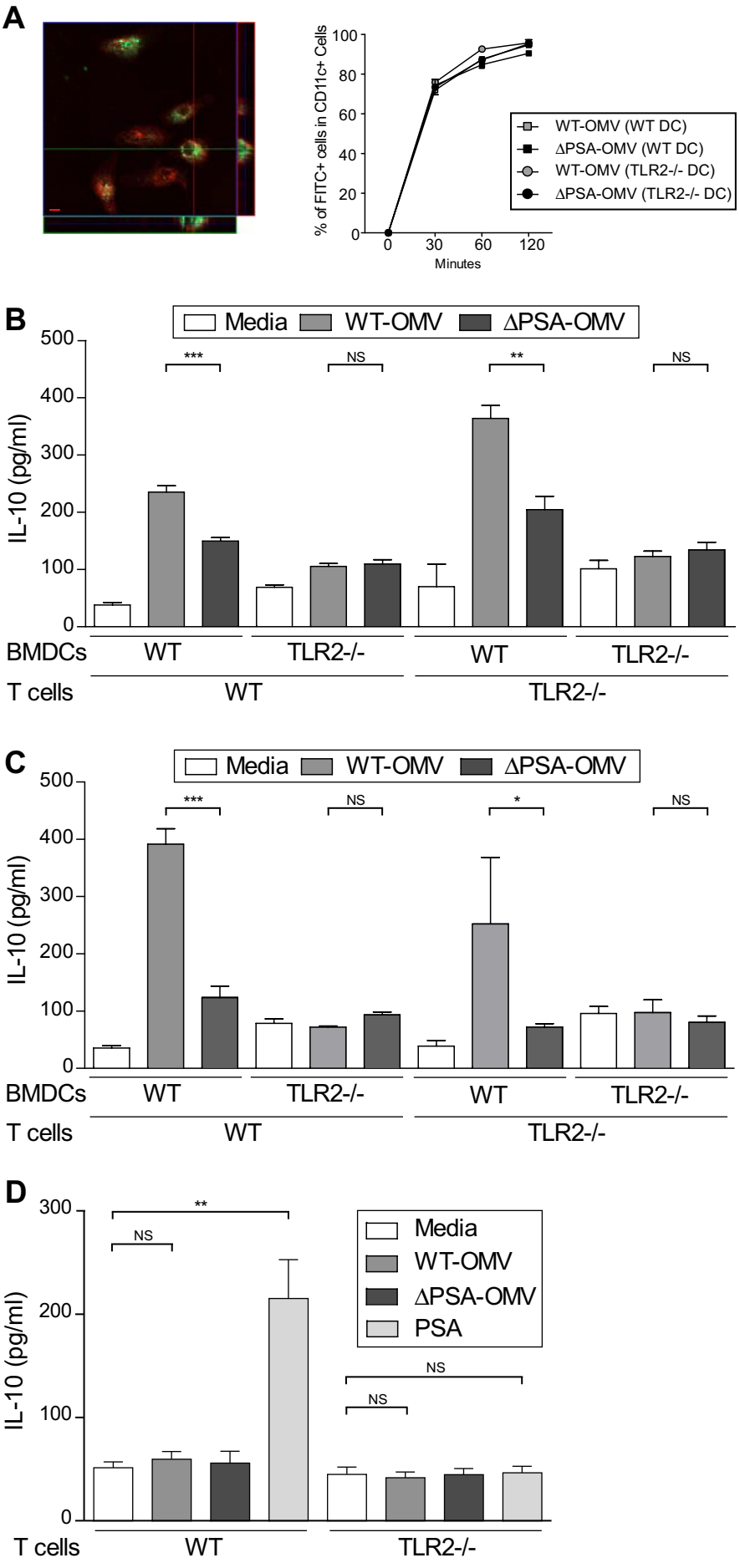
Shen *et al.*, Supplemental Figure 2

Figure S2, related to Figure 2. PSA-containing OMVs given orally to animals suppress pro-inflammatory cytokines and induce anti-inflammatory cytokines in colon tissue during TNBS colitis. (A) Cytokine transcript analysis by qRT-PCR of RNA recovered from purified CD4+ T cells from mesenteric lymph nodes. Each symbol represents a single animal. Error bars indicate SEM from 4 animals/group. Results are shown from 3 combined experiments, each performed independently. **(B)** Cytokine transcript analysis by qRT-PCR from RNA recovered from whole colons of each treatment group. Each symbol represents a single animal. Error bars indicate SEM. Results are shown from 3 combined experiments, each performed independently.**(C)** Cytokine analysis by intracellular cytokine staining (ICCS) on CD4+ T cells from colon LPL preparations of each treatment group. Each symbol represents a single animal. Error bars indicate SEM. Results are representative of 2 independent trials.**(D)**Weight loss in animals (treated with OMVs rectally) following the induction of TNBScolitis (day 0) measured as reduction from initial weight until day of sacrifice (day 3). All groups contained 3-4 animals, with error bars indicating standard error(SEM). Results are representative of 2 independent trials. **(E)** Quantification of colon lengthfrom vehicle treated (EtOH) and TNBS groups. Error bars indicate SEM. *** $p<0.001$. NS: notsignificant.Results are representative of 2 independent trials.



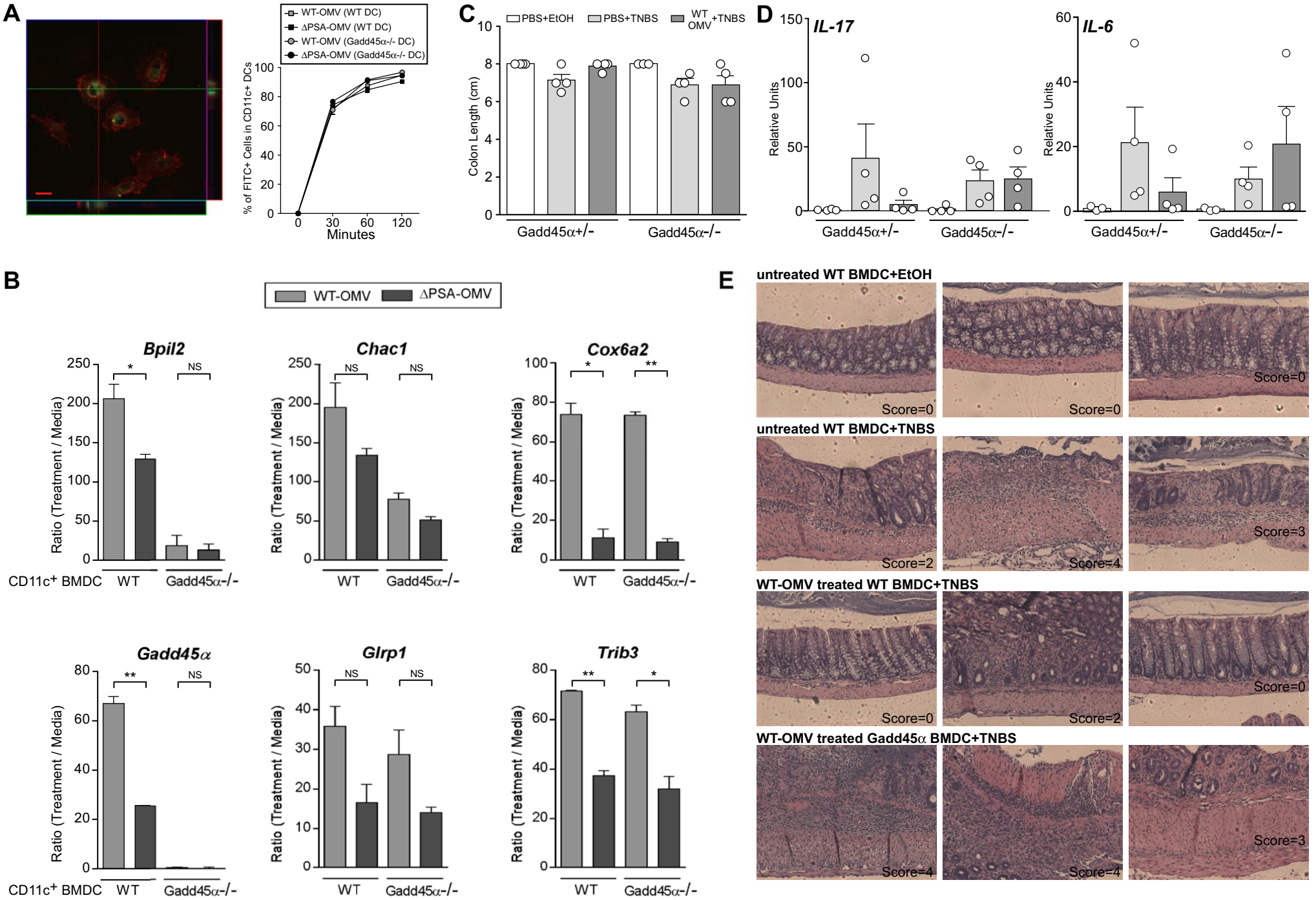
Shen *et al.*, Supplemental Figure 3

Figure S3, related to Figure 3. OMVs are internalized and localized in the cytoplasm of DCs, and up-regulate co-stimulatory molecules; neutralization of IL-10R signaling partially abrogates PSA activity *in vitro*. (**A**) Fluorescent micrographs of OMV (WT or ΔPSA) internalization by DCs. OMV were labeled with Fluorescein isothiocyanate (FITC, green) and incubated with cultured DCs for 2hrs. Cells were fixed and cell membrane was stained with Wheat Germ Agglutinin (WGA)-tetramethylrhodamine (red). Scale bar: 7.5μm. (**B**) Actin polymerization is required for OMV uptake by DCs. Flow cytometry analysis of OMV internalization by DCs pre-treated with Cytochalasin D. OMVs were labeled with FITC and incubated with cultured DCs for various times (as indicated). Cells were stained with anti-CD11c. Percentages show CD11c+OMV+ populations (compare to Figure 3A). (**C**) WT-OMVs and ΔPSA-OMVs up-regulate the co-stimulatory molecule CD86 (B7.2) for DC activation. FC plots of DCs incubated with WT-OMVs and ΔPSA-OMVs for various times (as indicated) and stained with anti-CD11c and anti-CD86. Percentages show CD86+ populations among CD11c+ cells. (**D**) Quantification of percentage of CD4+ T cells in each proliferating peak (as is labeled in Figure 3H). Error bars indicate SEM. Results are representative of 3 independent trials. * $p<0.05$; ** $p<0.01$; *** $p<0.001$. (**E**) Neutralization of IL-10R signaling partially abrogates PSA activity. *In vitro* suppression assay was set up as in Figure 3H except that CFSE labeled responder cells (Teff) were incubated with 20μg/ml of anti-IL-10R (+) or isotype control (-) for 1 hour before addition of Tregs purified from DC-T culture under various conditions as indicated. Percentages show total proliferating cells. (Treg:Teff=1:4) Results are representative of 2 independent trials.



Shen *et al.*, Supplemental Figure 4

Figure S4, related to Figure 4. TLR2-/- DCs internalize OMVs, and purified PSA elicits IL-10 production from T cells in a TLR2-dependent manner, while PSA-containing OMVs do not induce IL-10 directly from T cells. (A)(left)Fluorescent micrographs of OMV internalization by TLR2-/- DCs. OMV were labeled with Fluorescein isothiocyanate (FITC, green) and incubated with cultured DCs for 2hrs. Cells were fixed and cell membrane was stained with Wheat Germ Agglutinin (WGA)-tetramethylrhodamine (red). Scale bar: 7.5μm.(right)Quantification of FITC-labeled OMV uptake over time by wild-type DC or TLR2-/- DCs assessed by flow cytometry. **(B)** ELISA analysis for IL-10 production from culture supernatants of DC-T cell co-cultures, where DCs were pulsed with OMVs for 18 hours, washed and incubated with primary CD4+ T cells. Supernatants were collected at day 4 of culture. Media samples indicate DCs that were not pulsed with OMVs, but otherwise treated identically. Error bars indicate SEM from quadruplicate samples. Results are representative of 2 independent trials.**(C)** ELISA analysis for IL-10 production from culture supernatants of DC-T cell co-cultures exposed to OMV or media control. Supernatants were collected at day 4 of culture. Error bars indicate SEM from quadruplicate samples. Results are representative of 2 independent trials.**(D)** ELISA analysis for IL-10 of culture supernatants from T cell cultures exposed to OMVs or purified PSA. Supernatants were collected at day 4 of culture. Media samples indicate T cells that were not stimulated, but otherwise treated identically. Anti-CD3 was coated on the culture plate to activate T cells. Error bars indicate SEM from quadruplicate samples. Results are representative of 2 independent trials.



Shen *et al.*, Supplemental Figure 5

Figure S5, related to Figure 5. Gadd45α-/- DCs internalize OMVs, and Gadd45α is required in BMDCs to mediate PSA activity during protection from intestinal inflammation. **(A)** (left)Fluorescent micrographs of OMV internalization by Gadd45α-/- DCs. OMV were labeled with Fluorescein isothiocyanate (FITC, green) and incubated with cultured DCs for 2hrs. Cells were fixed and cell membrane was stained with Wheat Germ Agglutinin (WGA)-tetramethylrhodamine (red). Scale bar: 7.5μm.(right)Quantification of FITC-labeled OMV uptake over time by wild-type DC or Gadd45α-/- DCs assessed by flow cytometry. **(B)** Transcript analysis by qRT-PCR in FACS sorted wild-type and Gadd45a-/- CD11c+ BMDCs of selected genes that were induced by PSA-containing OMVs in a TLR2 dependent manner from microarray studies. Error bars indicate SEM of 4 samples. Results are representative of 2 independent trials. **(C)** Quantification of colon lengthfrom vehicle treated (EtOH) and TNBS groups of Gadd45α+/- mice and Gadd45α-/- mice. Error bars indicate SEM.Results are representative of 2 experiments performed independently. **(D)** Cytokine transcript analysis by qRT-PCR from RNA recovered from whole colons of each treatment group. Each symbol represents a single animal. Error bars indicate SEM. Results are representative of 2 experiments performed independently.**(E)** Images from hematoxylin and eosin (H & E) stained colon sections representative of each treatment group. Colitis scores from animals were assigned by a blinded pathologist (G.W.L) according to a standard scoring system (see Experimental Procedures).